Molecular characterization of *Myriogenospora atramentosa* and its occurrence on some new hosts

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Myriogenospora atramentosa produces partial to complete sterility of host grasses. Geographical and host species distributions were assessed and updated. It is reported only from the New World and appears to be limited to the highly evolved grass tribes Andropogoneae and Paniceae. Included in these tribes are the previously unreported host species *Panicum scoparium*, *Paspalum urvillei*, *Erianthus brevibarbis*, *E. contortus*, *E. giganteus*, and hybrids from crosses with *Erianthus* spp. Additionally, *M. atramentosa* is reported for the first time growing on *Paspalum notatum* (bahiagrass), and *Panicum hemitomon* in South Carolina, U.S.A. Sequence data (ITS regions 1 and 2 and 5·8S rDNA) from several isolates indicated that there is a significant level of DNA sequence differentiation between isolates of *M. atramentosa*, which suggests that there are at least two distinct sequence groups, or cryptic species, within the currently defined morphological species.

A condition in which the leaves of grasses, such as bahiagrass (*Paspalum notatum* Flugge), are bound to each successive leaf, resulting in a tangled arrangement and referred to as 'tangle top' disease, is caused by *Myriogenospora atramentosa* (Berk. & M. A. Curtis *apud* Berk.) Diehl. The fungus is a culturable biotroph of perennial warm-season grasses. Luttrell & Bacon (1977) placed the fungus in the tribe Balansieae (Clavicipitaceae), which contains taxa that produce ergot alkaloids and cause toxicity in cattle (Bacon *et al*., 1975, 1986; Bacon, Porter & Robbins, 1981). Cattle grazed on bahiagrass infected with *M. atramentosa* periodically show a stagger syndrome similar to paspalum staggers (Bacon, 1994), but the aetiology of this condition has not been identified. Determining the aetiological agent of bahiagrass staggers is usually complicated by the co-occurrence of an infection by *Claviceps paspali* F. Stevens & J. G. Hall, which produces tremorgenic metabolites (Cole *et al*., 1977) that may be responsible for this syndrome. Preliminary data have indicated that culture filtrates of *M. atramentosa* are toxic to chicken embryos (C. W. Bacon, unpublished). In addition to vertebrate toxicity, Clay, Hardy & Hammond (1985) reported that lepidopteran larvae fed *M. atramentosa*-infected leaves showed significantly reduced growth, survival, and weights in comparison with larvae fed non-infected leaves.

A deleterious effect on the host caused by this fungus is the reduced seed production due to the mechanical binding of the flag leaf to the emerging seed head by the fungal reproductive stroma. The extent to which the seed head is completely encased within a stroma varies, but some grasses are completely sterilized. Nevertheless, this fungus–grass association, as well as the majority of those in the Balansieae, is considered mutualistic, and several associations are being exploited biotechnologically (Funk *et al*., 1983; Bacon & Siegel, 1988). Information is, therefore, needed on the occurrence and distribution of Balansieae-infected grasses which may serve as additional sources of new grass–fungus combinations and assist in defining the biological significance of these associations in nature.

Presently, demonstrating that there are *Myriogenospora*–grass specificities is not possible since procedures for infecting grasses have not been determined (Rykard, Luttrell & Bacon, 1982; Rykard, 1983), and there is no information on the genetic variation within the species. Ribosomal DNA sequence analysis has been used to clarify the systematics of a group of related organisms (Glenn *et al*., 1996), and similar techniques have been used to study genetic variation within the family (Panaccione, 1996; Kuldau *et al*., 1997). The aim of the present study was to present an updated host and geographical range for *M. atramentosa*, and, using a PCR-based approach, to establish the similarities or differences among this species occurring on different hosts.

MATERIALS AND METHODS

Myriogenospora-*infected plants and fungus culture*

Symptomatic plants (Table 1) were observed or collected during late summer (August–September), and some were transplanted and maintained in either a greenhouse or field

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Table 1. Host range and geographical distribution of *Myriogenospora atramentosa*

Host	Location	Reference
Andropogon scoparius	LA	(Diehl, 1934)
A. scoparius	NC	(USDA, 1970)
A. virginicus	GA	(Luttrell & Bacon, 1977)
A. virginicus	SC*	
Axonopus compressus	PR	(USDA, 1970)
Cymbopogon citratus	FL	(Sprague, 1950)
Eragrostis hirsuta	GA	(Luttrell & Bacon, 1977)
Eremochloa ophuroides	GA	(Rykard, 1983)
Erianthus brevibarbis†	LA, SC	
E. contortus†	GA	
E. giganteus†	FL, GA	
Imperata brasiliensis	Brazil	(Hanlin & Tortolero, 1990)
Panicum anceps	VA	(USDA, 1970)
P. anceps	GA	(Luttrell & Bacon, 1977)
P. hemitomon	FL	(USDA, 1970)
P. hemitomon	SC*	
P. purpurascens	FL	(USDA, 1970)
P. scoparium†	SC	
Paspalum setaceum (= *ciliatoflium*)	VA	(USDA, 1970)
P. conjugatum	PR	(USDA, 1970)
P. conjugatum	Venezuela	(Hanlin & Tortolero, 1990)
P. dilatatum	GA	(Luttrell & Bacon, 1977)
P. laeve	AL	(Atkinson, 1894)
P. laeve	GA	(Rykard, 1983)
P. notatum	GA, FL	(Luttrell & Bacon, 1977)
P. notatum	SC*	
P. urvillei†	SC	
Saccharum officinarum	Brazil	(Vizioli, 1926)
S. officinarum	LA	(Abbott & Tippett, 1941)

* First report for South Carolina.
† New host.

cultures. During the initial collection period, stromata from grasses were placed on inverted lids of plastic Petri plates, and ascospores were collected by active discharge onto the surface of corn meal–malt (CMM) agar supplemented with antibiotics (Bacon, 1989). Germinating ascospores and subsequent colonies were maintained at room temperature in paraffin-sealed Petri plates. Under these conditions, colonies usually developed within 6–12 wk. Isolates were eventually stored at room temperature on CMM agar without antibiotics.

The identification of *M. atramentosa* from the various hosts was based on the asci, ascospores and the appearance of perithecia embedded in regular files within linear stromata on leaves (Luttrell & Bacon, 1977; White & Glenn, 1994). *In vitro* characteristics were also examined for some collections. Identifications were aided by the appearance of 'tangle top' on several host species (Diehl, 1934; Luttrell & Bacon, 1977) along with other generalized host symptoms of dwarfing and suppression of inflorescences (Atkinson 1894; Diehl, 1934; Abbott & Tippett, 1941). As currently recognized, *Myriogenospora* contains only two species, *M. atramentosa* and *M. linearis* (Rehm) J. F. White & Glenn, which are easily differentiated by host-association and ascus morphology (White & Glenn, 1994).

Voucher specimens

Voucher specimens of species of the Balansieae and their non-infected hosts from South Carolina habitats were deposited in the Farlow Herbarium and include the following: *Atkinsonella hypoxylon* (Peck) Diehl on *Danthonia* sp., open woods, Lee State Park, Lee County; *Balansia epichloë* (Weese) Diehl on *Sporobolus indicus* (L.) R. Br., yard, Darlington County; *M. atramentosa* on *Paspalum notatum* Flugge, yard, Chesterfield County; on *Andropogon virginicus* L., yard, Chesterfield County; on *Panicum hemitomon* Schult., Prestwood Lake Bank, Darlington County; on *Panicum scoparium* Lam. (Figs 1–2), cool, moist pine tree stand, Lee County; and on *Paspalum urvillei* Steud., cool, moist, sandy bank of Lake Robinson, Chesterfield County.

Voucher specimens of *Erianthus giganteus* (Walter) P. Beauv. (along roadside, Madison County, Georgia, U.S.A.) and *E. contortus* Ell. (along roadside, Clarke County, Georgia, U.S.A.) infected with *M. atramentosa* were deposited in the Mycological Herbarium (GAM) of the University of Georgia (Figs 3–6).

Nucleic acid extraction

Isolates of *M. atramentosa* collected in 1994 and 1996 from *Paspalum notatum*, *Andropogon virginicus*, and *Erianthus contortus* were analysed as representative species for sequence comparison of nuclear ribosomal DNA (rDNA). For extraction of nucleic acids, isolates were either grown in M102 liquid medium (Rykard *et al*., 1982) on a rotary shaker (200 rpm) at room temperature until adequate growth occurred (usually 1–2 wk), or nucleic acids were extracted directly from the stroma which was removed from the plant by careful scraping. Cultured mycelium was collected by centrifugation, and the pelleted tissue was washed once with sterile distilled water to remove excess medium. The tissue was then ground in liquid nitrogen and stored at −80 °C until ready for nucleic acid extraction. Extractions were made from 0·2–0·3 g (wet weight) of ground mycelium. Stromal tissue removed from its host was ground directly in 400 µl of extraction buffer with a microcentrifuge tube pestle.

Extractions of nucleic acids were performed using a slight modification of the CTAB protocol of Graham, Mayers & Henry (1994). Fungal tissue was combined with CTAB extraction buffer [2% (w/v) CTAB; 100 mM Tris-HCl (pH 8·0); 1·4 M NaCl; 20 mM EDTA (pH 8·0); and 1% 2-mercaptoethanol] and incubated at 60° for 30–45 min. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, homogenized with sample, and the sample was then centrifuged at 15 000 ***g*** for 5 min at 4°. Upper aqueous phase was transferred to a new tube, and DNA was precipitated by adding 0·1 vol. of 3 M sodium acetate (pH 5·2) and 2 vol. of ethanol (−20 °). Samples were mixed by inversion and placed at −20° overnight. DNA was pelleted by centrifugation, washed once with 70% ethanol (−20°), dried, and resuspended in TE buffer.

Polymerase chain reaction and sequencing

For each DNA extraction, 10–100 ng of DNA was used as template for polymerase chain reactions (PCR) (Mullis &

Fallona, 1987; Saiki, 1988). For each amplification, a total reaction volume of 100 µl was made containing DNA template, 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 2·5 mM $MgCl_2$, 0·5 µM of each primer, 200 µM of each dNTP, and 2·5 units of AmpliTaq DNA polymerase (Roche Molecular Systems, Inc., Branchburg, NJ). Each reaction mixture was topped off with a thin layer of mineral oil and amplified using a Perkin–Elmer DNA Thermal Cycler 480 (Norwalk, CT).

Amplification of ITS-1, 5·8S rDNA, and ITS-2 was performed using primers ITS5 and ITS4. Cycling parameters for amplification consisted of an initial cycle with denaturation at 95° for 5 min, annealing at 51° for 1 min, and extension at 72° for 45 s. This cycle was followed by 38 cycles with denaturation at 95° for 45 s, annealing at 51° for 1 min, and extension at 72° for 45 s (plus 4 s addition to extension segment per cycle). A final cycle was performed with an extension segment of 72° for 10 min.

Amplified products were separated from unincorporated nucleotides and primers using minicolumns (Wizard PCR Preps, Promega Corp., Madison, WI, U.S.A.) following the manufacturer's protocol. Purified samples were sequenced by the Molecular Genetics Instrumentation Facility of the University of Georgia (Athens, GA) using an Applied Biosystems automated sequencer (model 373A, version 1.2.1). Primers ITS5, ITS4, ITS3, and ITS2 (White *et al.*, 1990) were used to sequence ITS-1, 5·8S rDNA, and ITS-2.

Data analysis

The rDNA sequences were aligned by direct examination in conjunction with the ClustalW multiple sequence alignment application within the editing and application software package SeqPup v. 0·6f (http://iubio.bio.indiana.edu/IUBio-Software+Data/molbio/seqpup/). Large gaps and alignment ambiguities were excluded from our analyses. Maximum parsimony analysis of the aligned sequences was conducted using PAUP v. 3.1.1 (Swofford, 1993) on a Macintosh Performa 6115CD. Alignment gaps were treated as missing data (GAPMODE = MISSING). Informative gaps were, however, included as additional characters in the data matrix (absence and presence of gaps were coded as 0 and 1, respectively). Heuristic searches were performed with the following options in effect: tree-bisection-reconnection (TBR) sampling algorithm, collapsing zero length branches, and saving all minimal length trees (MULPARS). Ten replications with random addition of taxa were performed for each heuristic search in order to find any additional islands of minimum length trees (Maddison, 1991). Characters were equally weighted, and character states were unordered. To measure the relative support and stability of the resulting clades, bootstrap values (Efron, 1982; Felsenstein, 1985) and decay indices (Bremer, 1988; Donoghue *et al.*, 1992) were calculated using PAUP v. 3.1.1. Bootstrapping was performed with 250 replications. Decay indices up to 10 steps longer than the most parsimonious tree were determined. *Atkinsonella hypoxylon* (GenBank accession no. U57405) and *Balansia obtecta* (accession no. U57402) were used as outgroup taxa based on the results of previous analyses (Glenn *et al.*, 1996) which were of a much broader taxonomic scale.

RESULTS AND DISCUSSION

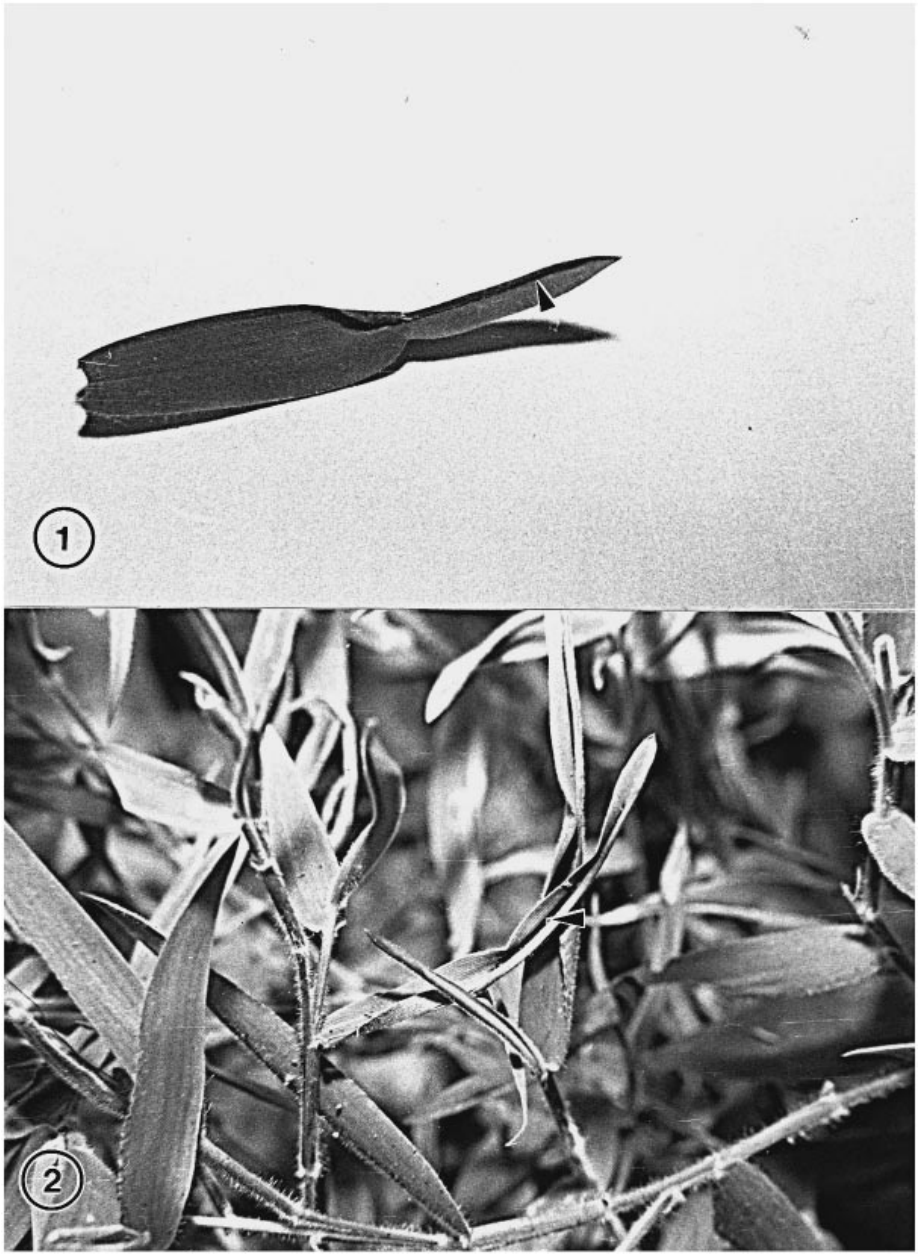
Host symptomatology and fungus-induced variation

Perennation of the disease is accomplished by hyphae overwintering in dormant buds, from which the fungus infects new emerging shoots the following year (Rykard, Bacon & Luttrell, 1985). The stroma usually occurs on the upper one-third adaxial portion of the leaf blade (Figs 1–6). On approximately 40% of the leaves of each bahiagrass tiller, *M. atramentosa* produces what is commonly referred to as the 'tangle top' disease of grasses (Luttrell & Bacon, 1977). This condition results from the tip of one leaf being bound to the distal region of another leaf by the stroma. This binding can occur successively as the leaves are produced and extend, thus producing a tangled arrangement of leaves (Fig. 3). The remaining leaves of a tiller may or may not be infected. Uninfected leaves are invariably the older leaves produced early in the season, apparently before the fungus was actively growing. On *P. scoparium* and other grass species, only a small percentage (< 10%) of the leaves are tangled. In these species the majority of leaves of a tiller will show untangled infections on the adaxial distal portions of leaves (Figs 1, 2).

The fungus first appears as an ephemeral, cream to pale brown conidial stroma in the folded centre or rolled portion of the blade (Rykard *et al.*, 1982). Conidia are produced holoblastically from sympodially proliferating conidiophores (Rykard *et al.*, 1982). The conidial stage is followed by perithecia formation which is indicated by the stroma becoming dark brown to black. Globose to subglobose perithecia are completely immersed within the epibiotic, linear stroma in regular files of one or two rows (Fig. 6) (Luttrell & Bacon, 1977; Hanlin & Tortolero, 1990; White & Glenn, 1994).

A major effect of this fungus on grasses is sterility. The few seeds that are produced on uncaptured inflorescences are viable, and the plants produced from these seeds are not infected by the fungus (Rykard *et al.*, 1985). Thus, this species may be viewed as pathogenic, although the benefits to the host may off-set losses derived from sterility (Bacon & Hill, 1996). Infection by *M. atramentosa* is transmitted through vegetative propagation of the host. The sterility caused by *M. atramentosa* may be a potential problem in intergeneric sugarcane hybrids produced from crosses with *Erianthus brevibarbis* Michx. and *E. giganteus* that are apparently susceptible hosts (Figs 3, 4; D. M. Burner, pers. comm.). These hybrid plants could become naturally infected, an event little understood even after several years of inoculation and infection attempts which failed to produce infected plants of wild species (Rykard *et al.*, 1985). Besides the reduction in seed, infected plants often show other symptoms of disease, although no quantitative measurements have been made. These include reduced tiller size and changes in tiller morphology for some species, for example, an increased number of adventitious roots and size of rhizomes in bahiagrass.

The non-invasive epibiotic infection of grasses by *M. atramentosa* is limited to the stromal interface with the cuticle (Luttrell & Bacon, 1977; White & Glenn, 1994). This association is in marked contrast to the intercellular habit of



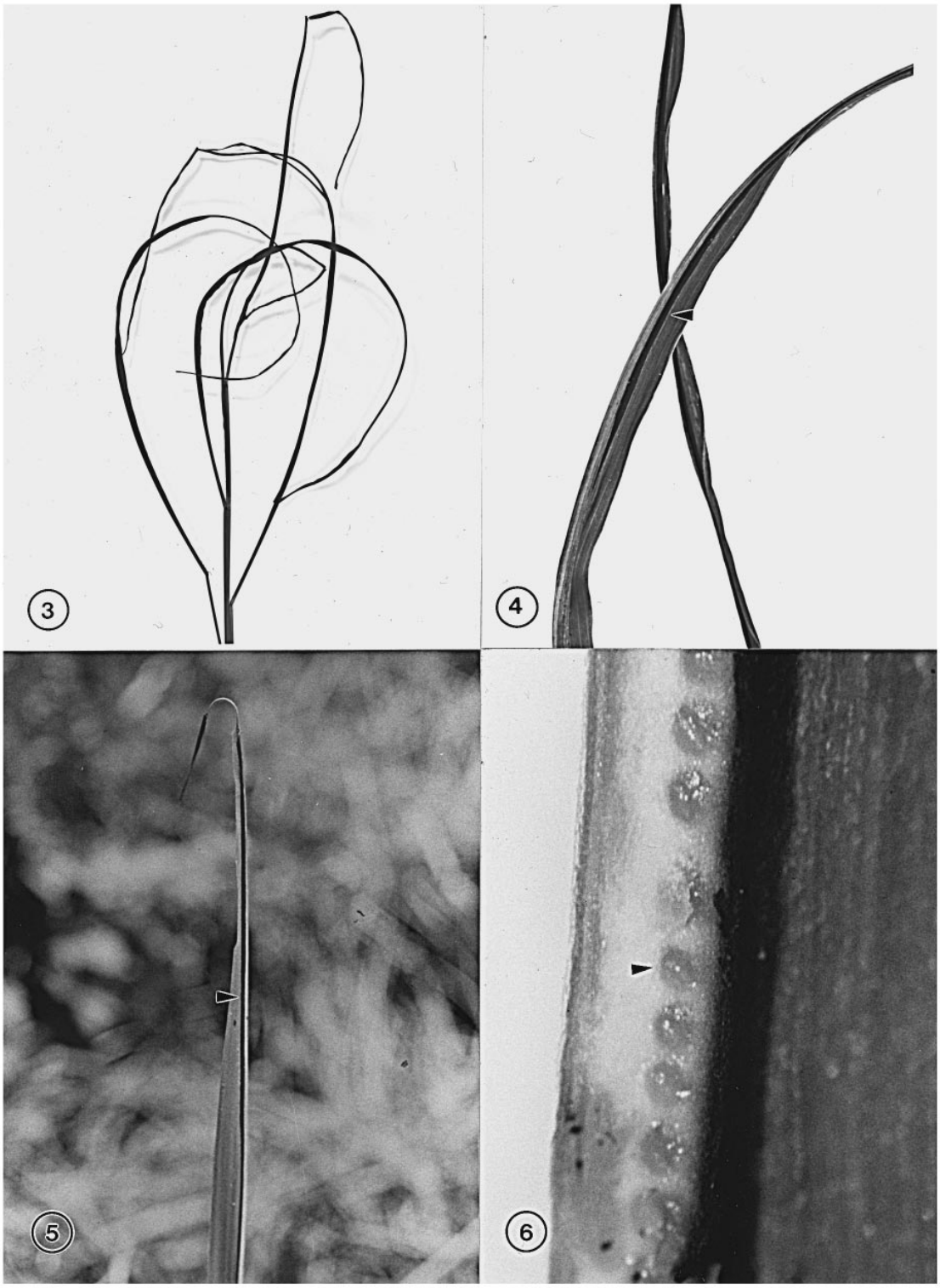
Figs 1, 2. *Myriogenospora*-infected *Panicum scoparium*. **Fig. 1.** Infected leaf showing rolled appearance of distal third of leaf due to binding by the stroma (arrow). **Fig. 2.** Infected grass showing black stroma (arrow).

other species of the Balansieae (White, 1994). Previously reported histological studies (Rykard *et al*., 1985; Smith, Bacon & Luttrell, 1985) indicated that the host epidermal cells beneath the fungus were enlarged and abnormally shaped. There were, however, no gross macroscopic alterations of the infected leaf, and no necrotic areas.

Host and geographical range

This is the first report of *M. atramentosa* in South Carolina, and the first report of it infecting *Panicum scoparium* (Figs 1, 2). The infected plants occurred near an artesian well, shaded by pine trees, and surrounded by *Myriogenospora*-infected *Paspalum notatum* in Lee County, South Carolina. Additionally this is the first report of this fungus on *Paspalum urvillei*. These infected plants were found on a shady slope of a lake in Chesterfield County, South Carolina, surrounded by infected *P. notatum* and *Andropogon virginicus*.

This is also the first report of *M. atramentosa* infecting species of *Erianthus* (Figs 3–6). Infected plants of *E. giganteus* and *E. contortus* were found along roadsides in Georgia within Madison and Clarke Counties, respectively. Both species were sympatric with infected plants of *A. virginicus*. Infections of *E. brevibarbis* and *E. giganteus* are reported in Louisiana (D. M. Burner, pers. comm.), as are infections of intergeneric hybrids (Abbott & Tippett, 1941) produced by crosses with *Saccharum officinarum* L. (sugarcane). *Erianthus* spp. and other wild relatives of *S. officinarum* are being used in an intergeneric sugarcane breeding programme in an effort to increase sugarcane resistance to sugarcane mosaic virus and sugarcane smut (*Ustilago scitaminea* Syd. & P. Syd.) (Grisham & Burner, 1992; Burner & Grisham, 1993). While sugarcane is a reported



Figs 3–6. *Myriogenospora*-infected *Erianthus* spp. **Fig. 3.** Infected collection from Georgia showing 'tangle top' disease on *E. giganteus*. **Fig. 4.** Black perithecial stroma (arrow) formed on a leaf that is rolled from one margin to the mid-vein of *E. giganteus*. **Fig. 5.** Linear, black stroma also formed on half-rolled leaf of *E. contortus*. **Fig. 6.** Longitudinal section through a stroma showing the globose perithecia (arrow) arranged in a single row on leaves of *E. contortus*.

host for *M. atramentosa* (Vizioli, 1926; Abbott & Tippett, 1941), such infections are apparently very rare (Sivanesan & Waller, 1986; and R. T. Hanlin, personal observation). It is possible that the apparent high susceptibility of *Erianthus* spp. to infection by *M. atramentosa* may have conferred greater susceptibility to sugarcane hybrids.

The fungus has been found on at least 21 species in 10 genera, but is only distributed among two tribes of the Gramineae (Table 1). These grasses include important forage species, as well as sugarcane. All species are warm-season perennial grasses with the C_4 NADP malate dehydrogenase type biochemical acid cycle (Hattersley, 1986), and the Kranz-

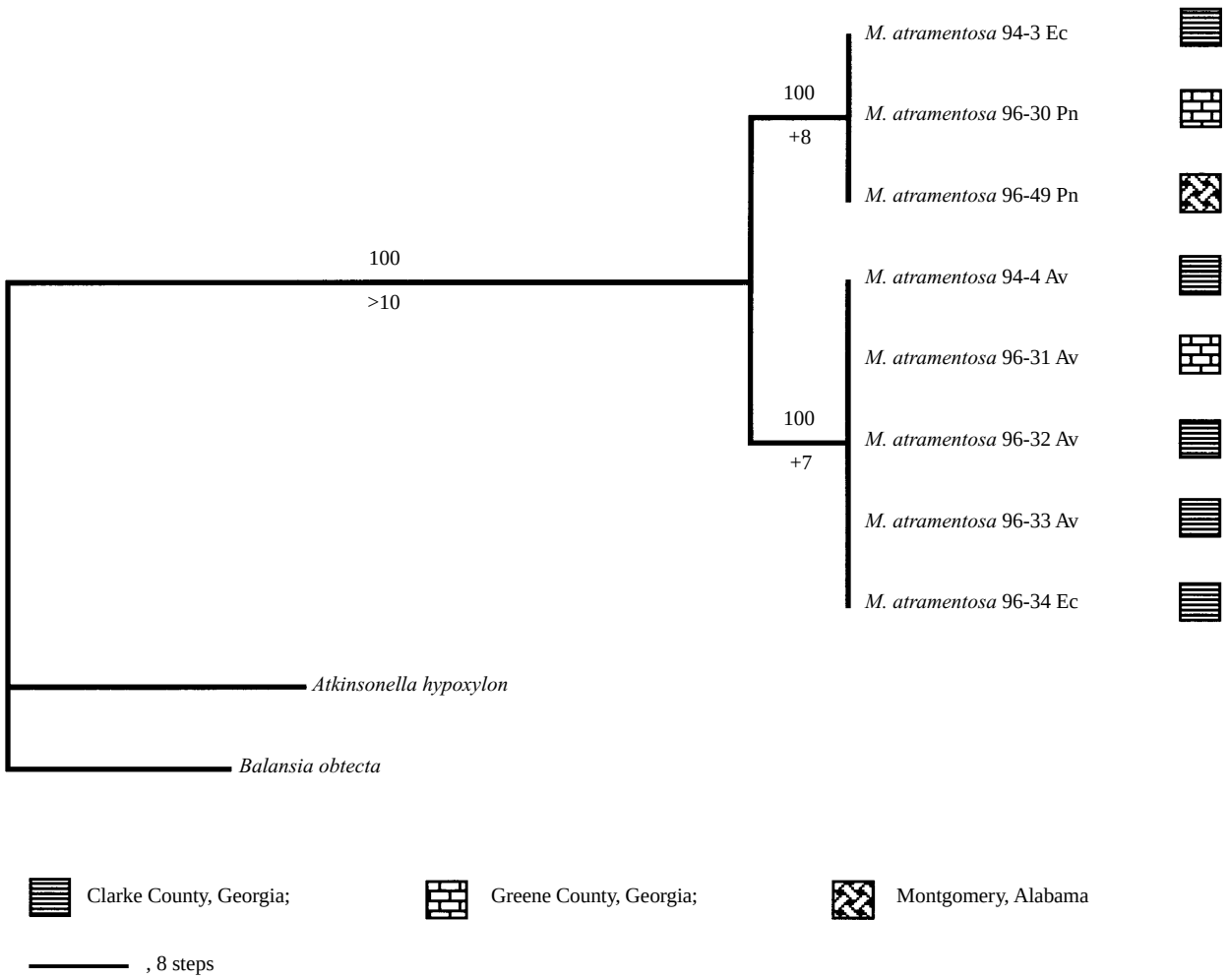


Fig. 7. Single most parsimonious cladogram of 118 steps. Bootstrap values and decay indices are given above and below the branches, respectively. Numbers following taxa are collection numbers. GenBank accession number for fungi within the top clade is U57406, and for those in the bottom clade it is U57407. Ec, *Erianthus contortus*; Pn, *Paspalum notatum*; Av, *Andropogon virginicus*.

type anatomy. *Myriogenospora atramentosa* has been reported only from the New World, occurring from the coastal areas of the southeastern United States inland from Virginia to Louisiana and south to Puerto Rico, Venezuela and Brazil.

Other members of the Balansieae found in South Carolina and surrounding southeastern states include *Balansia epichloë* on *Sporobolus indicus* (L.) R. Br. (smutgrass) (Rykard, 1983; Rykard *et al*., 1984), and *Atkinonsella hypoxylon* on *Danthonia* sp. (spring, 1985, unreported). The host and geographical range of *B. epichloë* has been reported (Diehl, 1950; Bacon *et al*., 1986).

rDNA sequence analysis

The resulting data matrix from the aligned sequences consisted of 653 bp and 14 additional characters in the form of informative alignment gaps. Of the 653 bp, 70 were excluded from the analysis because of alignment ambiguities. Individual sequences have been deposited into GenBank (see Fig. 7). The sequence alignment is available from A. E. Glenn upon request.

Maximum parsimony analysis yielded a single tree of 118 steps with a consistency index (CI) and retention index (RI) of 1·00. The cladogram is presented in Fig. 7 along with the resulting bootstrap and decay values. Bootstrap values for the three ingroup clades were all 100%. Branches of the cladogram do not begin to collapse until seven steps greater than the most parsimonious tree (118 + 7). Likewise, the number of most parsimonious trees remains to be just one tree up to seven steps greater (118 + 7), at which point the number of trees increases to 541 trees.

Two distinct groups of *M. atramentosa* isolates are evident in Fig. 7, and the isolates within each of these groups share sequences of 100% identity. As indicated by these sequence data, a cryptic differentiation appears to exist between the two groups and is partly observable in relation to host relationships. Geographically isolated collections of *M. atramentosa* from *P. notatum* have the identical sequences (100%) that are clearly distinct from the sequences of the two geographical collections made from *A. virginicus*. Host relationships are not, however, completely correlated with the resulting cladistic analysis. Two collections made from *E. contortus* at the same location in two different years each have different sequences and, therefore, appear in different clades. This observation suggests that both cryptic groups are capable of colonizing *E. contortus*, and both appear to exist in the same geographical area.

Sequence data from the isolates sampled clearly indicate that there is some level of DNA sequence differentiation between isolates of *Myriogenospora*. Our results indicate that

there are at least two distinct sequence groups within the currently defined species *M. atramentosa*. The distinction between these two groups is so strongly supported by statistical analyses of the sequence data (i.e. perfect bootstrap values and high decay indices), a possibility exists whereby there may be at least two cryptic species within the morphologically uniform *M. atramentosa*.

These results indicate that, within a geographical area, individual isolates from different cryptic groups can exist together, either on different hosts or on the same host. Also, members of each cryptic group show identical sequences to other members of the same group despite differences in geographical location and host grasses. The infected grasses sampled in this study all belong to two tribes, Andropogoneae and Paniceae, that are relatively closely related (supertribe Panicoideae), and are considered to be the most advanced and the most specialized of the grass tribes (Hattersley, 1986). Thus, *M. atramentosa* is associated with a relatively small group of grasses with very little phylogenetic variation (Sobral *et al*., 1994) and has evolved with two of the most advanced tribes of grasses, suggesting that its epibiotic habit may be derived from the endophytic habit common to most fungi in the Balansieae, contrary to an earlier suggestion that was based only on fungus–host morphology (Bacon & Hill, 1996). Other members of the Balansieae are associated with a much more diverse number of tribes, most of which are primitive, but with which the endophytic habit is considered a significant feature in their evolution.

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